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Cellulolytic and xylanolytic activities of common indoor fungi

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ABSTRACT

Mouldy building materials, such as chip wood and gypsum, should be a good source for fungal strains with high production of lignocellulolytic enzymes. Screening of 21 common indoor fungal strains showed, contrary to the expected, that the *Chaetomium* and *Stachybotrys* strains had little or no cellulolytic and xylanolytic activities using AZCL-assays. On the other hand, both *Cladosporium sphaerospermum* and *Penicillium chrysogenum* showed the highest cellulase, β-glucosidase, mannase, β-galactanase and arabinanase activities and would be good candidates for over-producers of enzymes needed to supplement or boost the bioconversion of lignocellulose-rich biomass.

1. Introduction

Filamentous fungi are among the most efficient degraders of plant biomass, whether it is undesired, as in the deterioration of Army Cotton Canvas, or by design, as in the bioconversion of organic waste material. They are, therefore, the main source of commercial lignocellulase production (Glass et al., 2013) and high yielding fungal strains are always in demand in the biotech industry (Pedersen et al., 2009; Hansen et al., 2015). The most commonly used organism for commercial enzyme production is *Trichoderma reesei* (Glass et al., 2013), but it has its limitations. *Trichoderma reesei* produces a high amount of exo-cellulases, but is a poorer producer of e.g. β-glucosidase (Okeke, 2014).

Research and screening of filamentous fungi for new high-yielding strains have shown that the original habitat of a strain is important in order to get as specific an enzyme profile as possible. Most of the commercial enzyme producers originate from habitats and substrates rich in lignocellulose, such as compost or agricultural soil (Hansen et al., 2015). Pedersen et al. (2008) showed that *Ulocladium* strains originating from cereal grain (starch) had the highest production of amylase compared to strains from the indoor environment (lignocellulose), which in return had the highest production of arabinanase.

Incidences of fungal deterioration of indoor environments are increasing and can now be seen in most of the western world (WHO, 2010). It is most obvious on lignocellulose rich materials, such as chip wood or plywood, where the fungal growth can be substantial. It has been shown that there are particular fungal associations between 1) *Cladosporium sphaerospermum* and plywood 2) *Stachybotrys chartarum* and gypsum board and 3) *Ulocladium alternariae* and wallpaper (Andersen et al., 2011). Mouldy building materials should therefore be a good source for discovery of fungal strains that either produce novel enzyme profiles or over-produce known desirable enzymes.

The purpose with this study was to screen and compare the ten most common fungal species from water-damaged buildings for their production of lignocellulases using *T. reesei* RUT C30 as a reference strain.

2. Materials and Methods

2.1. Fungal strains, media, growth conditions and enzyme extraction

Twenty-one common indoor strains and one reference strain were used in the study. The identity, source and geographic origin of all fungi are listed in Table 1. All fungal strains are held at the IBT culture collection at Department for Systems Biology, DTU, Denmark. To generate inoculum and check the identity, each strain was inoculated on Campbell's V8 juice agar (V8, Samson et al., 2010) and incubated for 7 days at 25 °C. For enzyme production a semi-solid wheat bran/sphagnum peat (WB/SP) medium was made containing (per kg): 256.25 g wheat bran (Finax, Denmark), 153.75 g sphagnum peat (Mosebrug, Denmark) and 590 g water. 40.0 g of WB/SP medium was placed in each 250 ml shake flask and autoclaved. Each fungal strain was inoculated by cutting 3 agar plugs (10 mm in diameter) with spores and mycelium from the V8 plate and transferring these to the shake flask with the WB/SP medium. The shake flasks were incubated at 25 °C and shaken twice a day manually for one week. The experiment was performed in triplicates (66 shake flasks in total).

Each shake flask was added 50 ml double distilled autoclaved water and shaken at 175 rpm overnight at 4 °C. Each extract was filtered through Miracloth into a 50 ml falcon tube and centrifuged at 10,000 g at 5 °C for 15 min. The supernatant (enzyme extract) was transferred to a clean 50 ml falcon tube and stored at 4°C prior to screening.

2.2. AZCL assay preparation and screening

Six different Azurine cross-linked (AZCL) substrates were used for screening: arabinan, arabinoxylan (wheat and birchwood), HE-cellulose, galactan and β-galactomannan (Megazyme, Bray, Ireland). For each AZCL assay plates were made containing (per 500 ml): 144 ml stock solution, 356 ml double distilled water, 7.5 g agarose (Litex, HSB 200 Protein grade) and 0.5 g AZCL substrate. The stock solution consisted of phosphoric acid (0.08 M) (Merck, Ortho-Phosphorsaure, 85 %), glacial acetic acid (0.08 M) (Merck, 100 %) and boric acid (0.08 M) (Merck) in double distilled water. To prepare the different AZCL assay plates, 200 ml double distilled water was added to the 144 ml stock solution. The pH was adjusted to 6 and double distilled water was added again to give a total volume of 500 ml. Agarose was added and the solution was autoclaved at 120 °C. The AZCL substrate was pre-soaked in 96 % ethanol for 10 min before use. When the agarose solution had cooled to approximately 65 °C, the AZCL substrate suspension was added while stirring. The agarose solution was poured into Petri dishes (90 mm in diam.) and when solidified, 8 wells (5 mm in diam.) were cut in the plates and stored at 4 °C.

For screening 35 µl of enzyme extract was added to each well of the six different AZCL assay plates. The plates were incubated for 24 h at 30 °C. The activity of each enzyme was measured as the radius of the zone of released azurine dye (the blue halo) around each application well. The radius was recorded and converted to area (mm^2).

2.3. β-glucosidase assay and screening

Screening for β-glucosidase activity was done using para-nitrophenyl-β-D-glucopyranoside (pNPG) 5 mM (Sigma Aldrich) as substrate in 50 mM sodium citrate (pH 4.8). The screening was carried out in a microtiter-plate format according to (Sørensen et al., 2011). A 10 µl volume of enzyme extract was added to 100 µl substrate in 1.5 ml Eppendorf tubes and incubated in a Thermomixer® comfort (Eppendorf) at 50 °C for 15 min. At the end of the reaction 60 µl of the reaction volume was transferred to a microtiter plate already containing 100 µl 1 M Na_2CO_3 for termination of the reaction. Absorbance at 400 nm was measured

in a plate reader (BioTek, EL800). Para-nitrophenol was used for preparation of a standard curve. One unit (U) of enzyme activity was defined as the volume of enzyme needed to hydrolyze 1 µmol of pNPG in 1 min. Background subtraction was prepared for each sample with 100 µl substrate at reaction temperature. Hereafter, 100 µl stop reagent was added to the Eppendorf tube then 10 µl enzyme for reaction time 15 min. 160 µl of the reaction mixture was then transferred to the microtiter plate and the absorbance was measured at 400 nm.

3. Results

The AZCL enzyme screenings of the 21 indoor strains were made from crude enzyme extracts from 7 day-old wheat bran/sphagnum peat (WB/SP) medium in solid state fermentations. The analyses showed that both *Chaetomium elatum* and *C. globosum* had low or no cellulase, mannase or galactanase activities (Fig. 1) with the exception of *C. globosum* (IBT 7029) that had an average mannase activity, compared to the highest activities (Fig. 1b). None of the strains of *Stachybotrys chartarum* or *S. chlorohalonata* showed any cellulase, mannase or β-galactanase activity (Fig. 1). *Penicillium chrysogenum* showed the highest cellulase and mannase activities (Fig. 1a and b), while *Cladosporium sphaerospermum* showed the highest galactanase activity (Fig. 1c). *Trichoderma*, including the reference strain (*T. reesei* RUT C30), had in general good cellulase and mannase activities, but very low galactanase activity (Fig. 1).

The β-glucosidase screening again showed that all the *Chaetomium* and *Stachybotrys* strains had a very low activity (0.008 - 0.154 U/ml) compared to the reference strain (*T. reesei* RUT C30) that had an activity of 0.513 U/ml (Fig. 2a). Compared to the high activities (7.823 - 7.653 U/ml) shown by *P. chrysogenum* and *C. sphaerospermum*, both *T. harzianum* and *T. reesei* (RUT C30) showed low activities (Fig. 2b).

122 The screening for xylanase production was done on xylan from both wheat and birch and gave similar
123 results (Fig 3a and b). It showed all ten *Chaetomium* and *Stachybotrys* strains as the low producers, while
124 *Aspergillus versicolor* and *T. harzianum* were the high producer for both types of xylanases (Fig. 3a and b).
125
126 The arabinanase screening, however, showed that neither *T. harzianum* nor *A. versicolor* had any activity,
127 while *P. chrysogenum* had the highest (Fig. 3c). Some of the *Chaetomium* and *Stachybotrys* strains had
128 average activities (e.g. *C. globosum* (IBT 7029) and *S. chlorohalonata* (IBT 40285)), while others showed no
129 arabinanase activity after growth on the WB/SP medium.
130
131 A Principal Component Analysis in Figure 4 of all the enzyme screenings shows that *Chaetomium* and
132 *Stachybotrys* (to the left) had similar enzyme profiles, with a general low activity of all tested enzymes.
133 *Aspergillus* and *Trichoderma* (at the bottom) also had similar profiles, both showing high xylanase activities,
134 average cellulase, β-glucosidase and mannase activities and low or no β-galactanase and arabinanase
135 activities. *Penicillium*, *Cladosporium* and to some extent *Ulocladium* (at the top) were similar in having
136 average to high activities of all enzymes tested.
137
138 Three strains, *C. globosum* (IBT 7029), *U. alternariae* (IBT 9058) and *T. harzianum* (41332) fell outside their
139 respective groups. *Chaetomium globosum* (IBT 7029) had, overall, a higher activity than the other
140 *Chaetomium* strains, while *U. alternariae* (IBT 9058) and *T. harzianum* (41332) had a lower activity
141 compared to their sibling strains.
142
143 **4. Discussion**
144 Wheat bran/sphagnum peat (WB/SP) medium was used since it has been shown to be superior in inducing
145 a broad variety of enzymes (Kolasa M. et al., 2014; Meijer M. et al., 2011). WB was also the medium of
146 choice in the study of Pedersen et al., (2009), where 50 *Ulocladium* strains were screened for enzyme

activity using the AZCL substrates. An attempt to grow the indoor fungi on a similarly composed medium with crushed chipboard/wallpaper instead of wheat bran/sphagnum peat resulted in no growth, probably because the fungi needed a higher water activity to grow on this medium than WB/SP.

The study of Pedersen et al. (2009) also showed that there was variation between strains of the same species. This was also seen in this study, for example, with the three *Penicillium chrysogenum* strains that showed similar enzyme profiles in most assays, except for mannose, where *P. chrysogenum* (IBT 30128) had no activity, while the other two strains had the highest activity.

The cellulolytic and xylanolytic activities of *Chaetomium* and *Stachybotrys* have been reported since the 1920s, where the fungi destroyed military equipment and other outdoor cotton fabrics (Greathouse and Ames, 1945; Domsch et al., 2007). The results in this study show, contrary to the expected, that the *Chaetomium* and *Stachybotrys* strains originating from water-damaged building materials showed little or no cellulolytic and xylanolytic activities using AZCL-assays. Even *C. globosum* (IBT 7029 = CBS 148.51 = USDA 1042.4), which has been used for material testing (Reese et al., 1950), showed only average activities in the AZCL assays. The fact that these fungi still are able to grow and destroy cellulose-rich building materials, suggests that they have only exo-enzymes and/or membrane bound endo-enzymes, since the AZCL assays screen for extracellular endo-enzymes (Vidal-Melgosa et al., 2015). Also the low β-glucosidase activity suggests that these enzymes are membrane bound or intracellular. Another reason could be that *Chaetomium* and *Stachybotrys* can utilize other carbon sources than cellulose in the building materials. Similar results were seen for *Trichoderma*. The low β-glucosidase activity for *T. reesei* in this study is in accordance with other studies (Okeke, 2014) and it has been suggested that this enzyme is membrane bound or intracellular (Kubicek et al., 2009).

171 The other indoor fungi have larger varieties and higher activities of endo-enzymes compared to
172 *Chaetomium* and *Stachybotrys*, which might explain their higher frequency on and lesser specificity for
173 water-damaged building materials (Andersen et al., 2011). *P. chrysogenum* showed the highest activities for
174 most of the screened enzymes, which might explain its occurrence on most damp indoor surfaces. *C.*
175 *sphaerospermum* showed a similar result to that of *P. chrysogenum*, however, this fungus is more
176 specialized and also associated with plaster and grouts in bathrooms, due to its ability also to tolerate high
177 fluctuations in humidity (McGinnis, 2007).
178 The hypothesis of this study, that mouldy buildings constitute a good source for high cellulase and xylanase
179 producers, was partly proven, but not with the fungal species expected. However, both *C. sphaerospermum*
180 and *P. chrysogenum* would be good candidates for over-producers of enzymes needed to supplement or
181 boost e.g. *T. reesei* in bioconversion of e.g. garden/park waste or other lignocellulosic biomass into bio-fuel.
182

183 **Acknowledgements**

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185 Buildings) and the Danish Council for Strategic Research (MycoFuelChem, Grant No. 11-116803) for the
186 financial support.
187

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224

225 Figure legends

226

227 **Fig.1.** Comparisons of enzyme activity for all 22 fungal strains for A: AZCL-HE-cellulose, B: AZCL-β-
228 galactomannan and C: AZCL-galactan.

229

230 **Fig.2.** Comparisons of enzyme activity for all 22 fungal strains for β-D-glucopyranoside. A: *Chaetomium* and
231 *Stachybotrys* strains compared with *T. reesei* (gray). B: *T. reesei* (gray) compared with *Trichoderma*,
232 *Ulocladium*, *Cladosporium*, *Aspergillus* and *Penicillium* strains.

233

234 **Fig.3.** Comparisons of enzyme activity for all 22 fungal strains for A: AZCL-arabinoxylan (wheat), B: AZCL-
235 arabinoxylan (birchwood) and C: AZCL-arabinan.

236

237 **Fig. 4.** Principal Component Analysis of all 7 enzyme activities and all 22 fungal strains. Arbitrary scales.

Figure

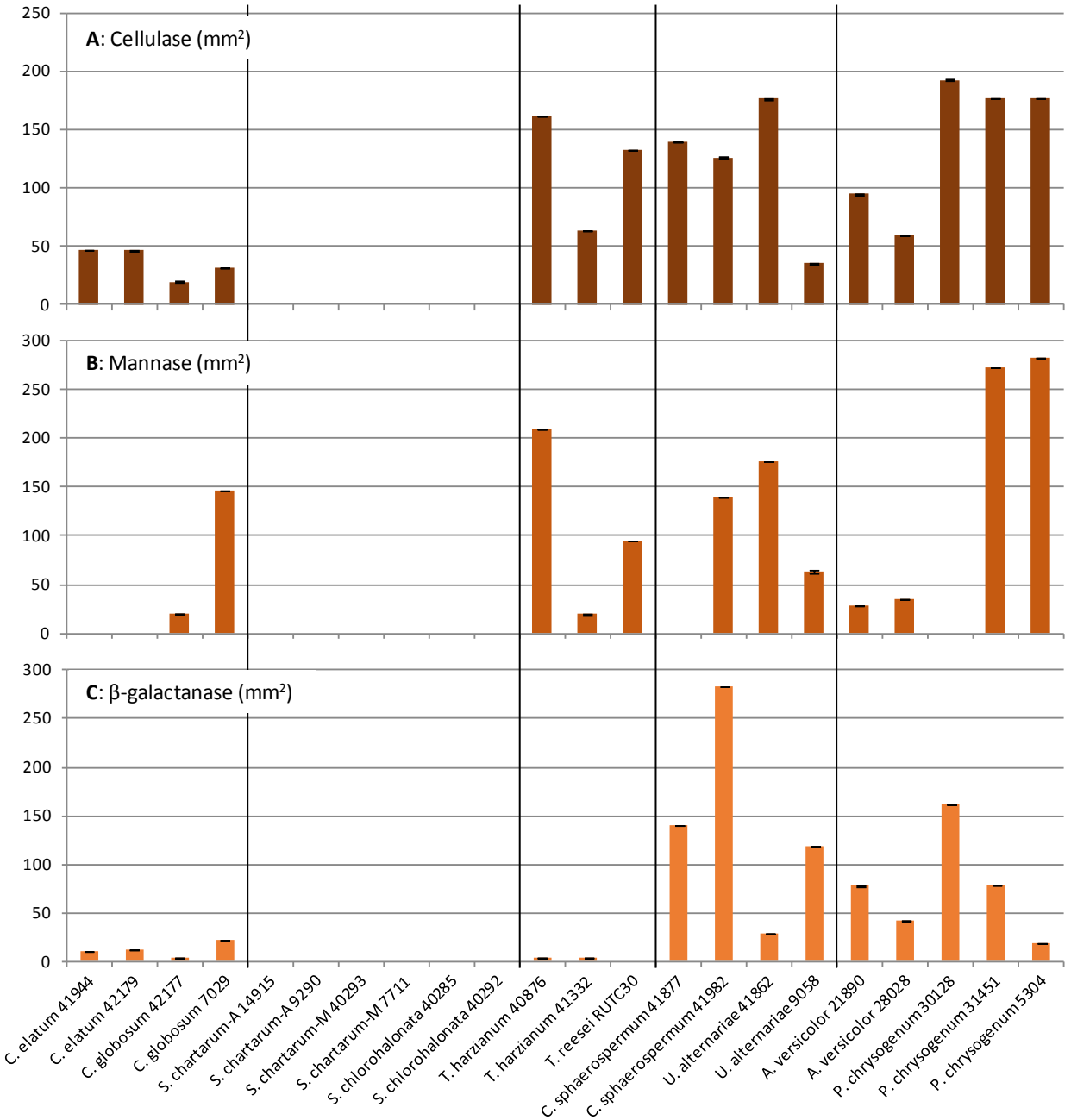

Figure 1

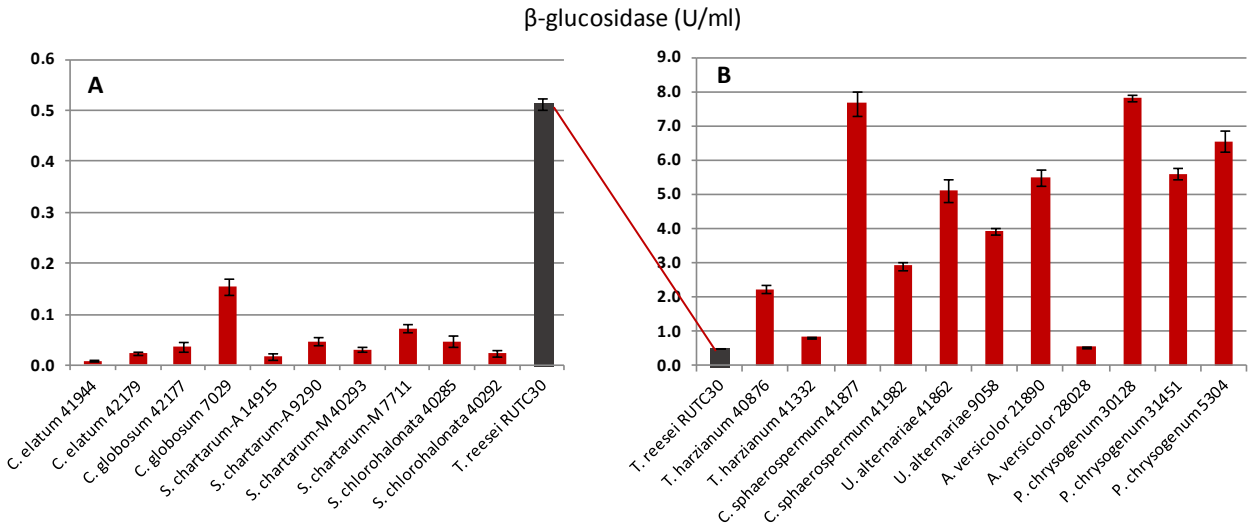
β-glucosidase (U/ml)
A
B
0.6
0.5
0.4
0.3
0.2
0.1
0.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
C. elatum 41944
C. elatum 42179
C. globosum 42177
C. globosum 7029
S. chartarum-A 14915
S. chartarum-A 9290
S. chartarum-M 40293
S. chartarum-M 7711
S. chlorohalonata 40285
S. chlorohalonata 40292
T. reesei RUTC30
T. reesei RUTC30
T. harzianum 40876
T. harzianum 41332
C. sphaerospermum 41877
C. sphaerospermum 41982
U. alternariae 41862
U. alternariae 9058
A. versicolor 21890
A. versicolor 28028
P. chrysogenum 30128
P. chrysogenum 31451
P. chrysogenum 5304

Figure 2

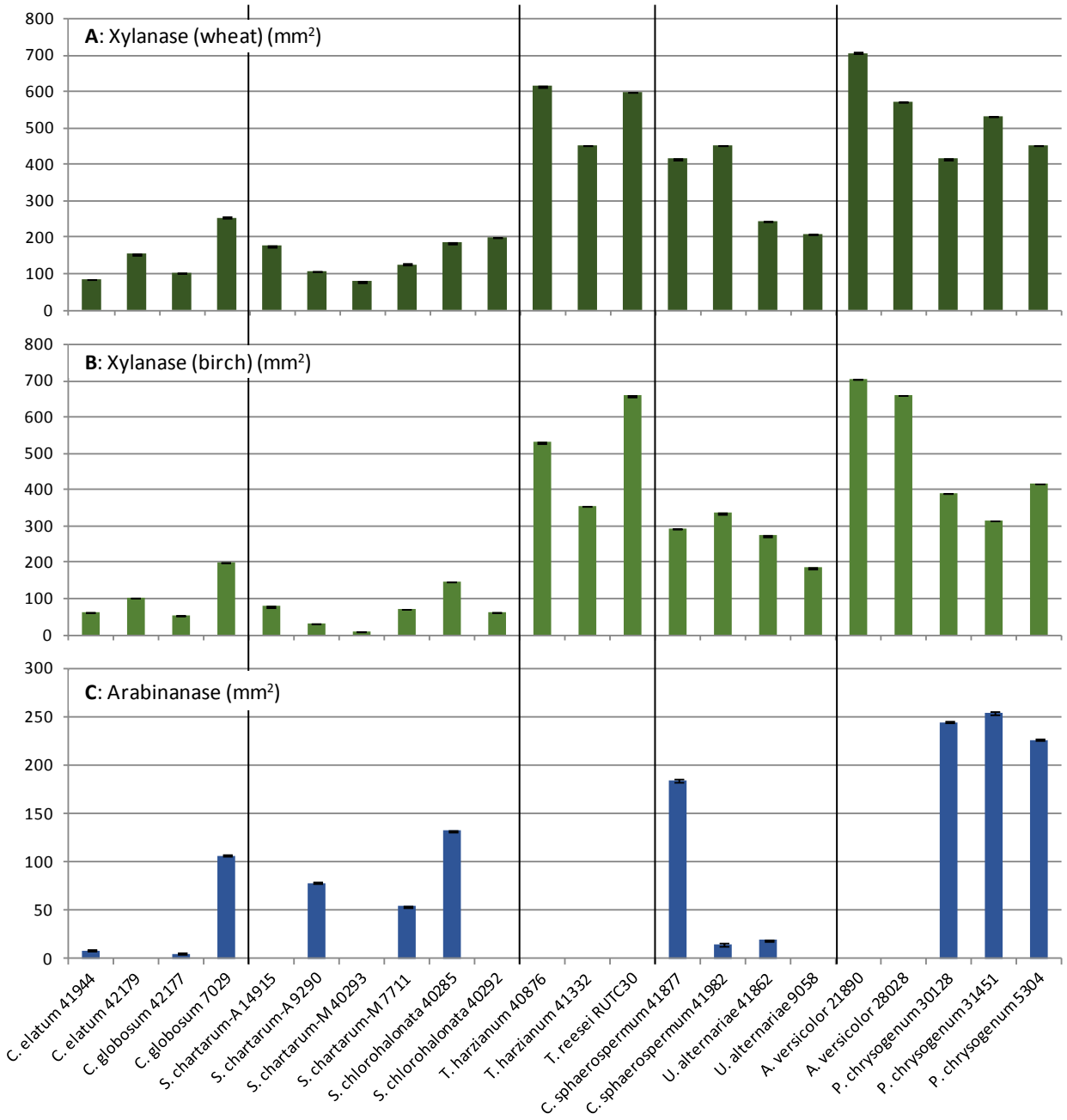
A: Xylanase (wheat) (mm²)
B: Xylanase (birch) (mm²)
C: Arabinanase (mm²)
C. elatum 41944
C. elatum 42179
C. globosum 42177
C. globosum 7029
S. chartarum-A14915
S. chartarum-A9290
S. chartarum-M40293
S. chartarum-M7711
S. chlorohalonata 40285
S. chlorohalonata 40292
T. harzianum 40876
T. harzianum 41332
T. reesei RUTC30
C. sphaerospermum 41877
C. sphaerospermum 41982
U. alternariae 41862
U. alternariae 9058
A. versicolor 21890
A. versicolor 28028
P. chrysogenum 30128
P. chrysogenum 31451
P. chrysogenum 5304

Figure 3

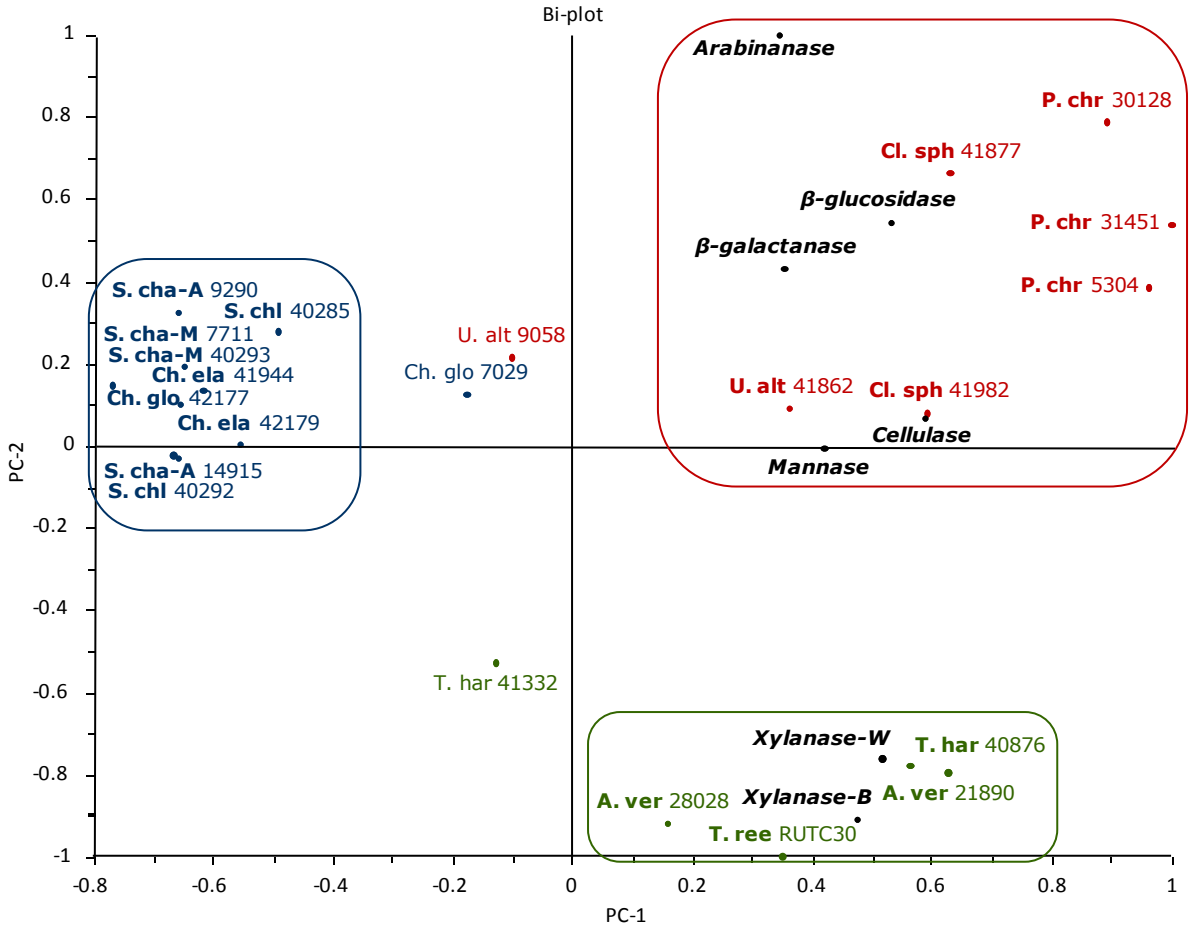
Bi-plot
PC-1
PC-2
Arabinanase
P. chr 30128
Cl. sph 41877
β-glucosidase
P. chr 31451
β-galactanase
P. chr 5304
U. alt 41862
Cl. sph 41982
Cellulase
Mannase
S. cha-A 9290
S. chl 40285
S. cha-M 7711
S. cha-M 40293
Ch. ela 41944
Ch. glo 42177
Ch. ela 42179
S. cha-A 14915
S. chl 40292
U. alt 9058
Ch. glo 7029
T. har 41332
Xylanase-W
T. har 40876
A. ver 28028
Xylanase-B
A. ver 21890
T. ree RUTC30

Figure 4

Table 1. Fungal strains used in this study with identification numbers and origin.

Genus	Species	IBT no.	Other no.	Origin
Aspergillus	*versicolor*	21890	KD 252-2	Indoor, USA
Aspergillus	*versicolor*	28028	NRRL 3499, SRRC 108	-, NL
Chaetomium	*elatum*	41944	BA Home A	Dust on curtain rail, DK
Chaetomium	*elatum*	42179	BA Sample 3009	Cardboard page from photo album, DK
Chaetomium	*globosum*	7029	CBS 148.51, USDA 1042.4	Stored cotton, USA
Chaetomium	*globosum*	42177	Krydsfiner X-a	Plywood, DK
Cladosporium	*sphaerospermum*	41877	BAV-KD-C1	Indoor air sample, DK
Cladosporium	*sphaerospermum*	41982	B221/914c	Pipe insulation, DK
Penicillium	*chrysogenum*	5304	LH 107	Indoor air sample, DK
Penicillium	*chrysogenum*	30128	DTO 78-E5	Indoor air sample, DK
Penicillium	*chrysogenum*	31451	GR11BA 10b-1-1b	Dust from vacuum cleaner, GL
Stachybotrys	*chartarum* (A)	9290	XX	Plaster wall, DK
Stachybotrys	*chartarum* (A)	14915	ALK 57	Gypsum board, DK
Stachybotrys	*chartarum* (M)	7711	Dyrup-J	Wood, DK
Stachybotrys	*chartarum* (M)	40293	201	Indoor, USA
Stachybotrys	*chlorohalonata*	40285	204	Indoor, USA
Stachybotrys	*chlorohalonata*	40292	103	Indoor, USA
Trichoderma	*harzianum*	40876	TMW 4.1880	-, -
Trichoderma	*harzianum*	41332	16534-a	Indoor air sample, DK
Trichoderma	*reesei*	-	RUT C30	-, -
Ulocladium	*alternariae*	9058	ALK 124	Indoor air sample, DK
Ulocladium	*alternariae*	41862	BA 1886	Wallpaper, DK